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## A mouse model of intestinal stem cell function and regeneration

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### SUMMARY

We present here an *in vivo* mouse model for intestinal stem cell function and differentiation that uses postnatal intestinal epithelial cell aggregates to generate a differentiated murine small intestinal mucosa with full crypt-villus architecture. The process of neomucosal formation is highly similar to that of intestinal regeneration. Both *in vivo* grafting and primary culture of these cells reveal two different epithelial cell populations, which display properties consistent with intestinal epithelial transit amplifying and stem cell populations.

Using this model system with a mixture of wild-type and transgene marked cells, we have shown that neomucosae originally develop from single aggregates, but that over time the mucosae fuse to form chimaeric mucosae. Despite fusion, the chimaeric mucosae maintain crypt clonality and villus polyclonality, demonstrating that clonal segregation persists during intestinal epithelial regeneration.

Key words: Small intestine, Stem cell, Organoid, Regeneration, Chimera, Mouse

### INTRODUCTION

The mammalian small intestine provides an elegant system for studying aspects of stem cell biology such as control of proliferation and differentiation. The mouse small intestine is lined with an epithelial monolayer, folded to form distinct crypt and villus structures, which are both physically and functionally distinct. The villi protrude into the intestinal lumen to absorb nutrients, whilst the crypts are hidden in protected pockets and function as the proliferative powerhouses of the intestine. Epithelial stem cells reside near the base of each crypt and divide to produce daughter cells, which migrate up towards the crypt mouth (Potten and Loeffler, 1990). As they do so these daughter cells, known as transit amplifying (TA) cells, proliferate rapidly and differentiate. The migrating cells appear at the mouth of the crypt as terminally differentiated cells and continue their path up towards the villus tip. Epithelial cells are then removed, possibly by a process involving apoptosis and release into the lumen (Hall et al., 1994; Shibahara et al., 1995). These events establish a stem cell hierarchy in which stem cells with maximum pluripotency and proliferative potential reside in the stem cell zone near the crypt base with cells lying above this zone displaying reduced pluripotency and proliferative potential, which reduces further as the distance from the stem cell zone increases (Potten and Loeffler, 1990). Throughout this process, specification of the four differentiated epithelial cell fates, rapid proliferation and cell migration must all be tightly regulated to ensure barrier integrity and intestinal homeostasis is maintained.

The crypts are derived ultimately from a single stem cell (Ponder et al., 1985), though there may be more than one

functional stem cell per crypt. The villi on the other hand are supplied with cells from a number of crypts and are polyclonal. Unlike other stem cell systems (Jones et al., 1995; Berardi et al., 1995), there are no available markers for intestinal stem cells. Furthermore, relatively little is known about the regulation of this multipotent population of cells, despite their known location (Hall and Watt, 1989). However, some information can be gleaned from various mouse mutants, particularly those which show increased susceptibility to intestinal neoplasia (for a review, see Stappenbeck et al., 1998). Little is also known about the signals controlling the morphogenesis of crypt and villus structures. In part, this is due to the limitations of model systems in which these processes can be studied. The use of organ and primary cultures (Fukamachi, 1992; Evans et al., 1992) is restricted because of their limited viability, which reduces the potential for experimentation. Xenograft systems using heterotypic recombinants of embryonic chick and rat tissue (Kedinger et al., 1981) or heterotopic recombinants of embryonic rat intestinal tissue (Duluc et al., 1994) have been used to show the instructive and permissive effects that both the intestinal endoderm and underlying mesenchyme have on intestinal epithelial development, morphogenesis and differentiation. However, these procedures rely on the increased developmental capacity of the undifferentiated endoderm and require a degree of skilful manipulation.

Stem cells are defined by their function and therefore in developing an assay for stem cells, it is first necessary to decide which properties satisfy this definition. The most important characteristics are considered to be the ability to (1) maintain a large proliferative capacity, (2) produce all epithelial lineages of the small intestine, and (3) recapitulate full crypt-villus

morphology, along with the maintenance of crypt clonality and villus polyclonality. However, isolation and manipulation of individual intestinal epithelial cells has so far failed to provide any functional evidence of stem cells. Clonal regeneration assays have been devised (Withers and Elkind, 1970; Potten and Hendry, 1975) whereby radiation is administered *in vivo* to kill off the majority of stem cells, allowing regeneration of the intestinal mucosa by surviving stem cells. These assays have provided insights into stem cell-mediated regeneration but are cumbersome and cannot be used to determine the identity of individual stem cells. The importance of the mesenchyme in regulating and maintaining endodermal differentiation would suggest that the mesenchyme has a role in regulating epithelial stem cell function. Tait et al. (1994) described a method in which aggregates of rat epithelial and mesenchymal intestinal cells are isolated and used to generate an intestinal neomucosa. We have modified and developed this technique and shown that cell aggregates from neonatal mouse small intestine can form two types of epithelial lined structures when grafted subcutaneously into immunocompromised mice. One structure appears to be derived from TA cells and the other from epithelial stem cells. We demonstrate that while the neomucosal epithelium is of donor origin, both donor and host cells contribute to the mesenchymal component and that mesenchymal-epithelial interactions are involved in crypt morphogenesis. We go on to show that it is also possible to generate a chimeric intestinal mucosa. These studies in a murine system confirm the utility and validity of the model in relation to intestinal regeneration and development. We also show the potential for its use in the study of interactions between different cell types.

## MATERIALS AND METHODS

### Isolation of mouse intestinal organoids

This technique was adapted from Evans et al. (1992) using rat small intestine. The small intestine was dissected from 6-12 neonatal CBA (Charles River) or ROSA26 (a kind gift from Dr Pen Rashbass) mice, aged 5-7 days and opened lengthwise. The tissue was cut into 0.5 cm fragments and these were washed 7-10 times in 1× HBSS (low calcium, low magnesium (Gibco-BRL), 2% D-glucose, 0.035% NaHCO<sub>3</sub>) to remove all luminal contents. The fragments were then finely chopped and digested in HBSS solution containing Dispase I neutral protease (Boehringer) at 100 µg/ml and crude collagenase type XI (Sigma) at 300 U/ml for 20 minutes at room temperature on a shaking platform. Digestion was stopped by the addition of 30 ml DMEM culture medium (Gibco-BRL) supplemented with 5% foetal calf serum (FCS), 2% sorbitol and penicillin and streptomycin at 60 µg/ml. Large fragments and muscle sheets were removed using gravity to allow them to settle to the bottom of the flask before removing the supernatant. Small fragments and single cells were removed by centrifugation of the supernatant for 3 minutes at 250 rpm to pellet the organoids. The supernatant was removed and the organoid pellet gently resuspended in 20 ml of the DMEM/sorbitol solution. The centrifugation step was repeated 5-6 times until the pellet contained a homogeneously sized organoid preparation.

### Subcutaneous grafting of mouse intestinal organoids

Mouse intestinal organoids were isolated as described and resuspended in a 2× volume of Matrigel (Collaborative Biomedical Products) or ECM gel (Sigma) supplemented with HGF (R&D Systems) at 50 ng/ml. A small incision was made on either side of the dorsal midline of a male scid mouse (Medical Microbiology, Edinburgh University),

and a subcutaneous tunnel created, into which 50-75 µl of the resuspended organoids were placed using a Gilson pipette.

### Retrieval of organoid grafts

The dorsal skin was cut from the mouse and the resulting skin flap examined. The grafted material could be identified as either large fluid-filled cyst structures, or small white lumps. These structures were carefully removed and placed into either 10% neutral buffered formalin for paraffin wax sectioning or into a screw-capped micro tube (Sarstedt) prior to immersion in liquid N<sub>2</sub> for cryostat sectioning.

### Primary culture of mouse intestinal organoids

Mouse intestinal organoids (approximately 1×10<sup>4</sup> per 60×15 mm culture dish (Falcon)) were isolated as described and cultured in high glucose DMEM (Gibco-BRL) supplemented with 10% FCS (Gibco-BRL) plus penicillin and streptomycin at 60 µg/ml each and incubated at 37°C in 5-10% CO<sub>2</sub>. The tissue culture medium was replaced after the first 24 hours, then after every 48 hours.

Organoids grown on Matrigel were cultured under identical conditions except that culture dishes were first coated with a thin layer of undiluted Matrigel. Organoids cultured within a thick layer of Matrigel were resuspended in Matrigel and placed in a tissue culture dish at 37°C to solidify before the addition of tissue culture medium.

### Immunohistochemistry

Tissues for immunohistochemical analysis were fixed overnight in 10% neutral buffered formalin and embedded in wax. Tissue sections (7 µm) were dewaxed and rehydrated prior to staining. Cultured cells were washed in PBS then fixed in 1:1 methanol:acetone for 5 minutes at 4°C prior to staining.

The following mouse monoclonal antibodies were obtained from Sigma. Anti-α smooth muscle actin, clone 1A4; anti-cytokeratin peptide 18, clone CY-90; anti-pan cytokeratin (mixture), clones C-11, PCK-26, CY-90, Ks-1A3, M20, A53-B/A2; anti-vimentin, clone VIM 13.2. A rabbit anti-lysozyme polyclonal antibody was purchased from Dako and an anti-PCNA antibody (PC10) was purchased from Santa Cruz Biotechnology Inc.

Antibodies were diluted in Tris buffer containing horse serum and applied to sections overnight at 4°C or at room temperature for 1 hour. The antibodies were detected using an immunoperoxidase system (Vectastain *Elite* ABC Kit, Vector Laboratories) according to the manufacturers' instructions.

### X-gal staining of cryostat sections

Staining of tissue sections for β-galactosidase activity using X-gal was always performed on cryosections of unfixed tissue. Tissues were quick-frozen in liquid N<sub>2</sub> then stored at -70°C until required. Tissues were embedded in OCT (Raymond A. Lamb) and sections typically 10-20 µm thick were cut from graft material. Tissue sections were washed in 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 20 mM MgCl<sub>2</sub> then fixed in wash solution containing 0.2% glutaraldehyde, 40 mM EGTA for 10 minutes at room temperature. Slides were washed for 3×5 minutes then stained in wash solution containing 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub> and X-gal at 1 mg/ml. Stained sections were post-stain-fixed in 4% paraformaldehyde in PBS at 4°C for at least 2 hours. Slides were washed in water, dehydrated and mounted.

### Histological staining of tissue sections

The Haematoxylin and Eosin (H&E) stain and the PAS stain were performed using standard protocols. The enteroendocrine cells are a heterogeneous population which produce a variety of products (Roth et al., 1990). The alkaline diazo stain (Wilson and Chalk, 1990) was used to identify a subpopulation of enteroendocrine cells that produce serotonin.

### Detection of alkaline phosphatase activity

Rehydrated tissue section or fixed cells were washed in PBS and



alkaline phosphatase activity was detected using the Vector® Red Alkaline Phosphatase Substrate Kit (Vector Laboratories), according to manufacturers' instructions, by a red precipitate.

## RESULTS

### Isolation and grafting of mouse small intestinal organoids

Epithelial organoids were obtained from neonatal (5-7 days) CBA mice, essentially as described by Evans et al. (1992). Briefly, this involves crude digestion of the small intestine using a mixture of collagenase and dispase. Intestinal aggregates, called organoids (Fig. 1A) were isolated from digested tissue using size fractionation steps to remove firstly large sheets of undigested cells and then single cells. The organoids were pelleted and resuspended in Matrigel or ECM gel supplemented with hepatocyte growth factor (HGF) prior to grafting to the subcutaneous dorsal plane of recipient scid mice. HGF was added since *in vitro* experiments had shown that it is capable of overcoming growth inhibitory effects of Matrigel/ECM gel on intestinal organoids (see below). Also, HGF has been shown to have angiogenic activity when implanted *in vivo* with Matrigel (Camussi et al., 1997). Grafts were retrieved at various time intervals and processed for paraffin wax sectioning. Sections were then examined histologically for evidence of intestinal mucosal development.

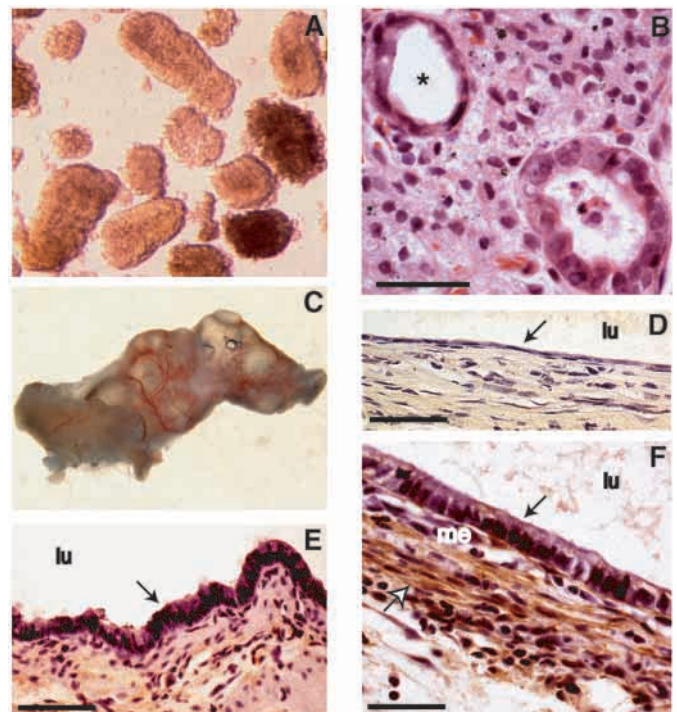
Small lumps of vascularised tissue were visible in 100% of all grafts. After 7 days, the tissue was composed primarily of stromal cells, within which epithelial-like cells could be identified surrounding a small lumen. At this stage 31% (8/26) of cysts were found to be lined with a homogeneous population of large, undifferentiated epithelial-like cells (Fig. 1B) and 27% of cysts (7/26) were lined with a layer of flattened epithelial cells (Fig. 1B). The remaining cysts (42%, 11/26) appeared to be lined with a more heterogeneous epithelium containing both large and flattened cells (data not shown).

By 9-14 days, cysts can be identified at the graft sites with the naked eye (Fig. 1C). Sections through these grafts reveal a variety of different epithelial morphologies lining the cysts. Undifferentiated flattened epithelium can still be identified (Fig. 1D) but the large undifferentiated epithelial cells are no longer present. Instead, differentiated columnar epithelium, similar to that found in intact small intestine, can be identified (Fig. 1E). In some cases these differentiated cells are found alongside the flattened epithelium within the same cyst, although the two cell types are never mixed. The epithelium is usually surrounded by a single layer of fibroblast-like cells, which in turn is surrounded by multiple layers of smooth muscle cells identified using an antibody directed against  $\alpha$ -smooth muscle actin (Fig. 1F). This entire structure is surrounded by stromal cells. Many cysts, especially those lined with a flattened epithelium, appear swollen and are filled with fluid.

Cysts lined with columnar epithelium develop small indentations where a region of the epithelium appears to migrate into the underlying mesenchyme (Fig. 2A). At the same time stromal cells underneath this region penetrate through the underlying smooth muscle cells and surround the migrating epithelium. This epithelial migration continues to form distinct crypts (Fig. 2B). Unlike cysts lined with flattened

epithelium, the lumen of these cysts are filled with mucin and cell debris. The non-crypt epithelium remains surrounded by a layer of mesenchymal cells and smooth muscle cells but the epithelium of the crypts is surrounded by large numbers of mesenchymal cells. These regions appear very similar to the crypt regions of intact mouse small intestine (Fig. 2D).

As grafts are left for longer periods of time (2-4 weeks), the number of individual cysts decreases but their size increases. This, combined with the mixed epithelial morphology of many cysts, suggests that cysts are fusing together. Most are lined with differentiated, polarized columnar epithelium, although even at 4 weeks a small number of cysts lined with flattened and columnar epithelium remain. However, by this stage the majority have developed mature crypts and a proportion have also developed folds of monolayered epithelium, which protrude towards the lumen. This marks the early stages of villus development, and by 5 weeks villi are readily identifiable (Fig. 2C). The graft at this stage appears very similar to the mucosa of the mouse small intestine and is referred to as a neomucosa. After 11-14 weeks the majority of cyst lumen are filled with mucus and cell debris, which distorts the cyst



**Fig. 1.** (A) Isolated organoids from neonatal mouse small intestine. (B-D) Cysts generated by subcutaneous grafting of mouse intestinal organoids. (B) H&E-stained section through organoid grafts after 7 days. A cyst lined with flattened epithelium is shown on the left (\*), next to a cyst lined with layer of large epithelium (right). (C) Cysts retrieved from graft site after 2 weeks. (D-E) H&E staining showing the morphology of the epithelium lining grafts after 2 weeks. Black arrows point to the epithelial lining of the cyst. (D) Region of undifferentiated flattened epithelium. (E) Region of polarised columnar epithelium. (F) Mesenchymal organisation surrounding epithelium. Section through cyst showing columnar epithelium (arrow) surrounded by a layer of fibroblast-like cells (me), which are in turn surrounded by several layers of smooth muscle cells (open arrow), stained brown using an antibody directed against  $\alpha$ -smooth muscle actin. lu, lumen; me, mesenchyme. Bar, 20  $\mu$ m.

morphology making histological analysis of the epithelial architecture increasingly difficult.

### Identification of cell lineages within intestinal neomucosa

To determine whether the stem cells contained within the organoids have retained their capacity to differentiate into multiple lineages, the cyst epithelium was examined for the presence of the four main intestinal epithelial cell types. These are absorptive enterocytes, goblet cells, enteroendocrine cells and Paneth cells. Absorptive enterocytes can be identified by the presence of alkaline phosphatase activity within the apically localised brush border (Fig. 3A). These cells are never identified within the flattened epithelium but are first identified with the emergence of columnar epithelial cells. Similarly, the emergence of this epithelial phenotype is also associated with the differentiation of goblet cells (Fig. 3B) and of enteroendocrine cells (Fig. 3C). Finally, the Paneth cell lineage can be identified by their morphology and location at the base of crypts (Fig. 3D).

### Proliferation of epithelium within the neomucosa

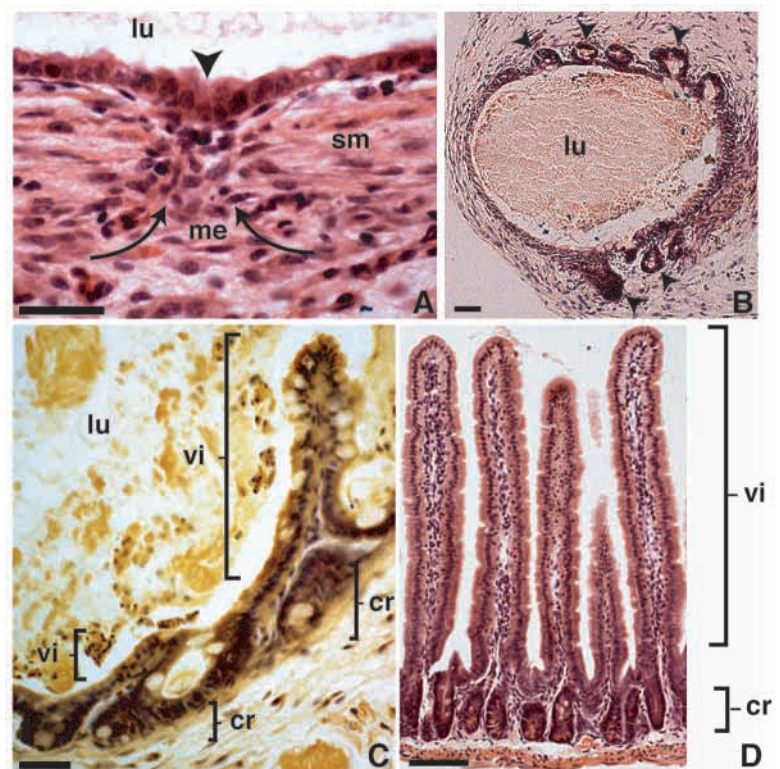
The TA cells of the small intestine are thought to be capable of only 4–6 rounds of cell division (Potten and Loeffler, 1990) while stem cells are thought to have a large and possibly infinite proliferative capacity. It is therefore important to show that active epithelial proliferation takes place within the neomucosal epithelium. Grafts retrieved after 31 days were stained for the presence of the proliferating cell nuclear antigen (PCNA), which identifies cells undergoing DNA replication (Kurki et al., 1986). In undifferentiated flattened epithelium displaying no crypt-villus architecture, only the occasional positively stained cell could be seen scattered throughout the

epithelium. Similar observations were made in cysts containing differentiated columnar epithelium. Clusters of positively staining epithelial cells can be identified in regions where the epithelium is beginning to migrate down into the mesenchyme (Fig. 4A). In more developed cysts, PCNA-positive cells become restricted to the newly formed crypts (Fig. 4B), as found in intact intestine. BrdU-labelling experiments showed that epithelial proliferation was still taking place at least 14 weeks after organoid grafting (data not shown).

### Grafting of intestinal organoids containing a transgene cell marker

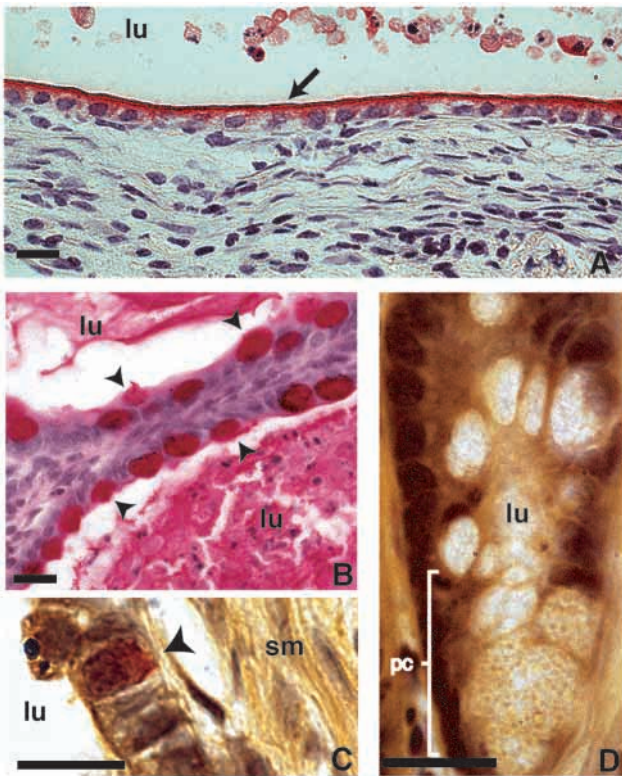
In order to ensure that the neomucosae in the grafts were indeed derived from donor tissue and also to determine the host contribution to the cysts, organoids containing an identifiable marker were grafted into scid mice. Organoids were obtained from neonatal ROSA 26 mice (Friedrich and Soriano, 1991), which ubiquitously express the gene encoding  $\beta$ -galactosidase in all cells of the intestine throughout intestinal differentiation and development (Wong et al., 1996). These transgene-marked organoids were resuspended in ECM gel and grafted into scid mice as previously described. Grafts were retrieved at 2 weeks and 16 weeks and sections were stained for  $\beta$ -galactosidase activity.

Analysis of grafted material revealed all the epithelium lining the cysts to be of donor origin as expected (Fig. 5A). However, stromal cells were a mixture of both donor and host origin, although the majority were derived from the host. The epithelium at all stages of neomucosal development stained blue, demonstrating that the  $\beta$ -galactosidase transgene was never switched off at any point. There were, however, some variations in the intensity of staining between cell types with the epithelium and smooth muscle cells staining more



**Fig. 2.** Neomucosal morphogenesis from organoid grafts. (A) Primary step in crypt morphogenesis. Section through cyst at 2 weeks stained with H&E reveals region of epithelium in the process of migrating down into the underlying mesenchyme (arrowhead). Stromal cells (me) from beneath the ring of smooth muscle (sm) can be seen migrating through the smooth muscle cells towards the epithelium in the direction of the arrows. (B) Crypt morphology in neomucosal development. H&E-stained section through cyst at 5 weeks showing the formation of multiple crypt structures (arrowheads) around the epithelial monolayer. (C) Villus formation in the neomucosa. Section through cyst at 5 weeks stained using the alkaline diazo method shows the formation of villus structures (vi) projecting towards the central lumen with crypts (cr) lying below the level of the villi. (D) H&E-stained section through adult mouse small intestine showing distinct crypt (cr) and villus (vi) structures. lu, lumen; me, mesenchyme. Bars, 20  $\mu$ m (A,C); 50  $\mu$ m (B,D).





**Fig. 3.** Identification of the four main differentiated intestinal epithelial cell types. (A) Apical localization of alkaline phosphatase activity, shown in red, identifies a population of absorptive enterocytes (arrow). (B) PAS staining reveals the presence of pink mucin-secreting goblet cells, examples of which are marked by arrowheads. (C) Alkaline diazo staining identifies a subset of enteroendocrine cells which produce serotonin. This can be visualised by the presence of red staining granules (arrowhead) in the basal region of epithelial cells. (D) Paneth cells (pc) can be identified by their location at the base of crypts and by their distinctive large granules. lu, lumen; sm, smooth muscle; ep, epithelium. Bars, 10  $\mu$ m.

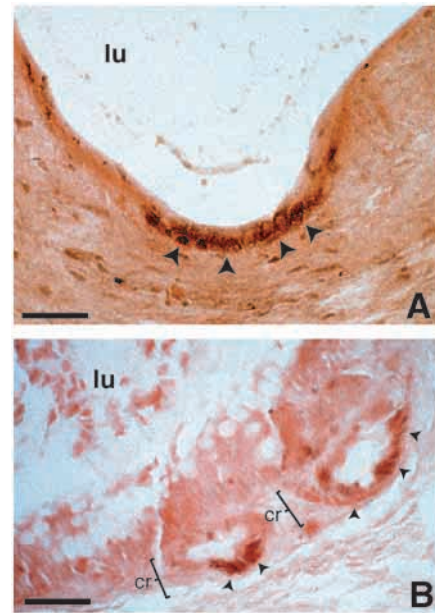
intensely. This staining pattern is equivalent to that seen in the small intestine of adult ROSA 26 mice (Fig. 5E).

### Generation of a chimeric neomucosa

In order to understand more about the process of neomucosal formation, a mixture of intestinal organoids obtained from both ROSA 26 and CBA animals were grafted together. Grafted material was retrieved after 3 and 11 weeks and stained for the presence of  $\beta$ -galactosidase activity.

At 3 weeks the majority of cysts were lined with epithelium derived either from the ROSA 26 or from the CBA organoids. This data suggests that at these early stages the cysts develop from individual organoids. A number of cysts developed with epithelium derived solely from CBA organoids but were surrounded by regions of stromal cells derived from ROSA 26 cells (Fig. 5B). This suggests that the donor mesenchymal contribution is not necessarily derived from the same organoid as the epithelium. It is more likely that individual mesenchymal aggregates from the original organoid preparation are recruited during neomucosal development.

Grafted material retrieved after 11 weeks showed the



**Fig. 4.** Cell proliferation in the developing neomucosa. (A) PCNA-positive cells (arrowheads) can be identified in regions of epithelium, which are beginning to migrate into the underlying mesenchyme at the onset of cryptogenesis. (B) Proliferative (PCNA-positive) cells (arrowheads) become restricted to the crypts (cr) in later stages of neomucosal development. Bars, 50  $\mu$ m.

majority of cysts contained a mixture of ROSA 26- and CBA-derived epithelium (Fig. 5C). Epithelium derived from each strain was found in neighbouring individual blocks rather than a random mixture of cells, confirming previous observations of cyst fusion. The crypts in these neomucosae were found to be derived from either CBA or from ROSA 26 cells (Fig. 5C) and crypts containing cells of both strains were never detected, even at the junction between epithelial patches from different strains. Villus structures were generally composed of cells derived from a single strain. However, villi situated at the boundary between epithelium derived from different strains were composed of cells from both strains (Fig. 5D). These results are identical to that seen in the intestine of chimeric mice (Schmidt et al., 1985; Wong et al., 1996), demonstrating that crypts are clonal whereas villi are polyclonal.

### Primary culture of mouse intestinal organoids

Many factors may account for the heterogeneity in epithelial development between grafts at 9 days and beyond. Differences in the time taken for organoids to connect to the host's blood supply, and therefore obtain access to circulating growth and survival factors, may influence the degree of neomucosal development. A large amount of data exists showing the importance of the extracellular matrix (ECM) on the differentiation program of intestinal epithelia (Kedinger et al., 1986; Sanderson et al., 1996; Duluc et al., 1997; Plateroti et al., 1997). Variations in the local extracellular environment of the organoids within the Matrigel/ECM gel may therefore influence development. A third possibility is that the proliferative and differentiation repertoire of individual organoids varies, so that cysts originating from different organoids produce a variety of different structures. To test the

latter possibility, freshly isolated intestinal organoids were cultured and the ability of cells from individual organoids to proliferate and differentiate was examined.

After 24 hours in culture the majority of organoids did not adhere to the culture dish and many organoid structures had dissociated into single cells. Approximately 1% of organoids, however, did adhere to the culture dish and these were grown in fresh medium to study their potential for proliferation and differentiation. Colonies were formed and antibodies directed against cytokeratins and vimentin were used to identify epithelial and mesenchymal cells, respectively. Immunohistochemistry confirmed the presence of both epithelial and non-epithelial colonies in the cultures, as would be expected from the initial heterogeneous cell population. Collating the data on the primary cultures from antibody staining, enzyme activity and cell proliferation showed that each epithelial colony could be categorised into one of 3 distinct types.

#### Type 1

A small number of colonies essentially comprise the original organoid and show little or no epithelial proliferation; however, some vimentin-positive mesenchymal cells could be seen proliferating out from the base of the organoid (data not shown). Alkaline phosphatase activity could be detected only in the cells

of the organoid, reflecting their differentiated status, and not in the surrounding mesenchymal cells (Fig. 6A). All cells appeared negative for cytokeratin 18, which is preferentially expressed in the undifferentiated crypt epithelium of the rodent intestine (Calnek and Quaroni, 1993; Flint et al., 1994).

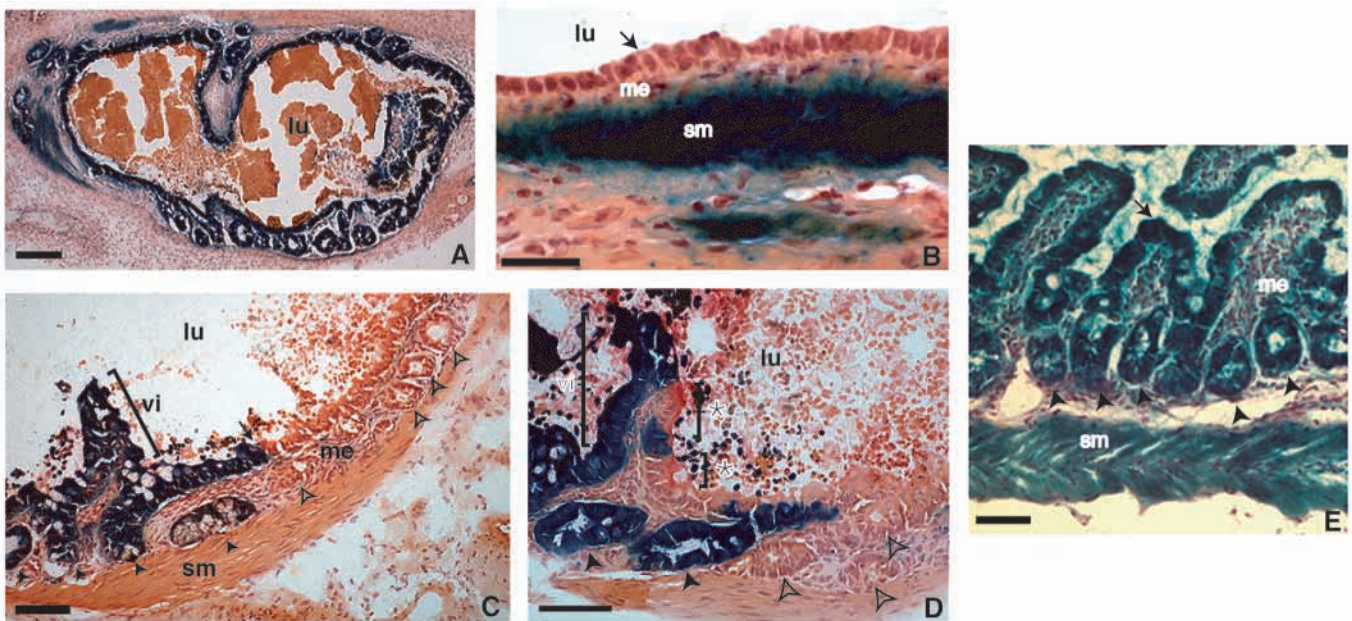
#### Type 2

These made up the vast majority of epithelial colonies in which cells proliferated rapidly for 2-3 days before proliferation ceased abruptly. The colonies were positive for alkaline phosphatase activity (Fig. 6B) and negative for cytokeratin 18. Surrounding the colonies were a number of mesenchymal cells negative for both markers.

#### Type 3

Approximately 1-2% of colonies exhibited a large capacity for proliferation which lasted for up to 2 weeks, producing a large sheet of epithelial cells. These cells were found to be negative for alkaline phosphatase activity but positive for cytokeratin 18 (Fig. 6C). Again, these colonies were surrounded by a number of mesenchymal cells.

To assess the effect of Matrigel on intestinal organoids we cultured them either on top of a thin layer of Matrigel or within a thick layer. In both cases, no cell proliferation could be



**Fig. 5.** (A) Identification of donor-derived cells within the neomucosa. X-gal staining of ROSA 26-derived neomucosa reveals all the epithelia lining the cyst lumen (lu) to be donor-derived (blue/black staining), whereas the surrounding mesenchyme is a mixture of donor and host derived cells. (B) Generation of chimeric neomucosa. Cyst generated from a mixture of ROSA 26 and CBA cells reveals that ROSA 26-derived smooth muscle cells (sm) are capable of associating with CBA-derived epithelium (arrow) with an intervening layer of host or CBA-derived mesenchyme (me). (C) Cyst containing both ROSA 26- and CBA-derived epithelium suggesting crypt fusion during cyst development. An arrow denotes the boundary between cells derived from the two different strains. Crypts within the chimeric neomucosa are derived either from ROSA 26 cells (solid arrowheads) or CBA cells only (open arrowheads). Crypts containing epithelial cells from both mouse strains are never detected, suggesting that these crypts are monoclonal. A ROSA26-derived villus (vi) can also be seen within the region of ROSA26 epithelium. (D) A villus (vi) located at the border of a patch of ROSA 26-derived epithelium and CBA-derived epithelium. X-gal staining reveals the presence of epithelial cells from both ROSA26 (blue/black) and CBA (\*) mouse strains present on the villus, thus demonstrating the polyclonal origin of this structure. Surrounding crypts are either exclusively ROSA26-derived (solid arrowheads) or CBA-derived (open arrowheads). Note that individual cells shed into the lumen are also derived from both mouse strains. (E) A section of ROSA26-derived small intestine after X-gal staining shows that while all cells are stained blue, there is variation in the intensity of the stain between cell types. The epithelia of the villi (arrow) and crypts (arrowheads), for example, are stained much more intensely than the mesenchymal cells (me). Abbreviations as in Fig. 2. Bars, 100  $\mu$ m (A); 20  $\mu$ m (B); 50  $\mu$ m (C,D,E).



detected even after 5 days. This may have been due to the presence of TGF $\beta$  within the Matrigel (Vukicevic et al., 1992) which has been shown to have a growth inhibitory effect on intestinal epithelium (Barnard et al., 1989). We therefore repeated the experiment, this time adding HGF to the tissue culture medium. This growth factor has been shown to counteract the inhibitory effects of TGF $\beta$  (Santos and Nigam, 1993) on epithelial morphogenesis. Organoids grown on top of a layer of Matrigel were now able to proliferate and produced colonies as described previously though the epithelial junctions appeared much tighter than when grown on plastic (Fig. 6D). The most dramatic effect was seen with organoids cultured within a thick layer of Matrigel, which formed cyst structures composed of an epithelial layer surrounding a central lumen (Fig. 6E). These structures resembled the early cyst structures found in the allograft experiments.

To assess the importance of cell-cell and cell-ECM interactions in neomucosal development, organoids cultured for 24 hours were removed from the culture dish, embedded in ECM gel either as single cell suspensions or as intact aggregates, and grafted subcutaneously into scid mice. Retrieval of grafted cells revealed that cyst development was only possible from cells grafted as aggregates (data not shown).

## DISCUSSION

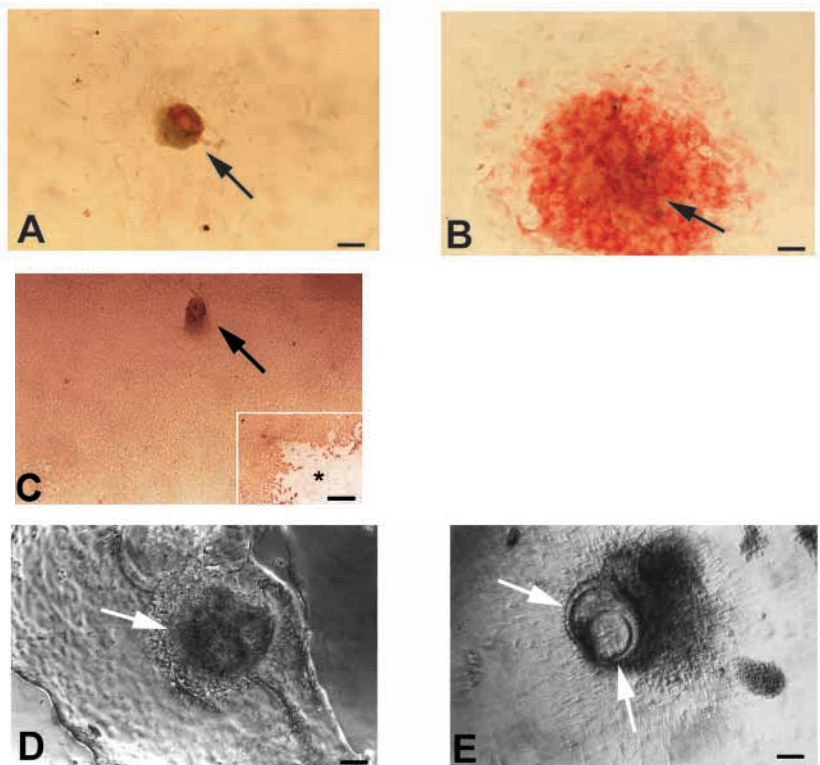
The small intestinal mucosa is an intricate, precisely organised structure composed of both epithelial and mesenchymal tissues, which interact to produce a functional and regulated organ. Models in which to study the formation and regulation of such a system need to replicate the complex interactions

between cell types. The system we describe here provides a technically simple and functionally accurate model in which to study intestinal stem cell function and mucosal regeneration.

Intestinal cell aggregates containing both epithelial and mesenchymal cells (Tait et al., 1994) were isolated. Grafting of these aggregates led to the development of an intestinal mucosa. Characterisation of this neomucosa revealed that it maintains all the key features of the normal intact intestinal mucosa. Using a mixture of transgene marked and unmarked cells, we have shown that neomucosae form initially from single organoids, but then fuse together as they increase in size to generate a chimeric neomucosa. However, the fusion of cysts does not cause mixing of the cells and the neomucosa resembles the intestine of chimeric mice with epithelium from individual strains found in distinct patches. All neomucosal crypts are derived from a single mouse strain whereas villi at the boundary of patches are composed of cells from both mouse strains. This makes it possible to distinguish and compare mutant tissue directly with wild-type tissue, even on the same villus, in a manner similar to that used successfully in chimeric mice (Hermiston et al., 1993; Crossman et al., 1994; Hermiston and Gordon, 1995). Our system has the advantages of being less time consuming, however, and allows simple control over the degree of chimerism within the neomucosa by altering the ratio of input organoids.

Understanding the developmental process of neomucosal formation from grafted organoids to differentiated mucosa will provide information on the process of regeneration and the establishment of the intestinal stem cell hierarchy. We have developed a model for neomucosal generation, based on the *in vivo* and *in vitro* data presented here (Fig. 7). Isolated organoids contained only two types of proliferative epithelial

**Fig. 6.** Primary culture of mouse intestinal organoids. (A) Type I colony stained for alkaline phosphatase activity (red) after 5 days in culture. Cells which make up the original organoid (arrow) stain positive, reflecting their differentiated epithelial phenotype, but surrounding fibroblast-like cells are negative. (B) Type II colony stained for alkaline phosphatase activity after 4 days in culture. Cells of the original organoid (arrow) and the newly generated surrounding epithelial cells are positive, reflecting their differentiated phenotype. These cells are negative for cytokeratin 18 and proliferation within these colonies ceases after approximately 48 hours. (C) Type III colony stained for cytokeratin 18 (brown), a marker for undifferentiated crypt epithelium, after 8 days in culture. All cells including those of the original organoid (arrow) are positive. Proliferation within these colonies continues for up to 2 weeks in culture. These cells are negative for alkaline phosphatase activity, confirming their undifferentiated phenotype. The inset shows the periphery of the same colony, demonstrating the contrast between positive epithelial cells and the non-staining fibroblasts (\*) surrounding the colony. (D) Effect of growing organoids on top of a Matrigel layer in the presence of HGF. A confluent sheet of tightly packed epithelial cells proliferate out from the organoid (arrow). (E) Organoids grown within a surrounding layer of Matrigel generate cyst-like structures composed of a central lumen lined by a layer of epithelial cells (arrow). Bars, 100  $\mu$ m (A-B); 150  $\mu$ m (C; inset, 200  $\mu$ m); 50  $\mu$ m (D); 100  $\mu$ m (E).



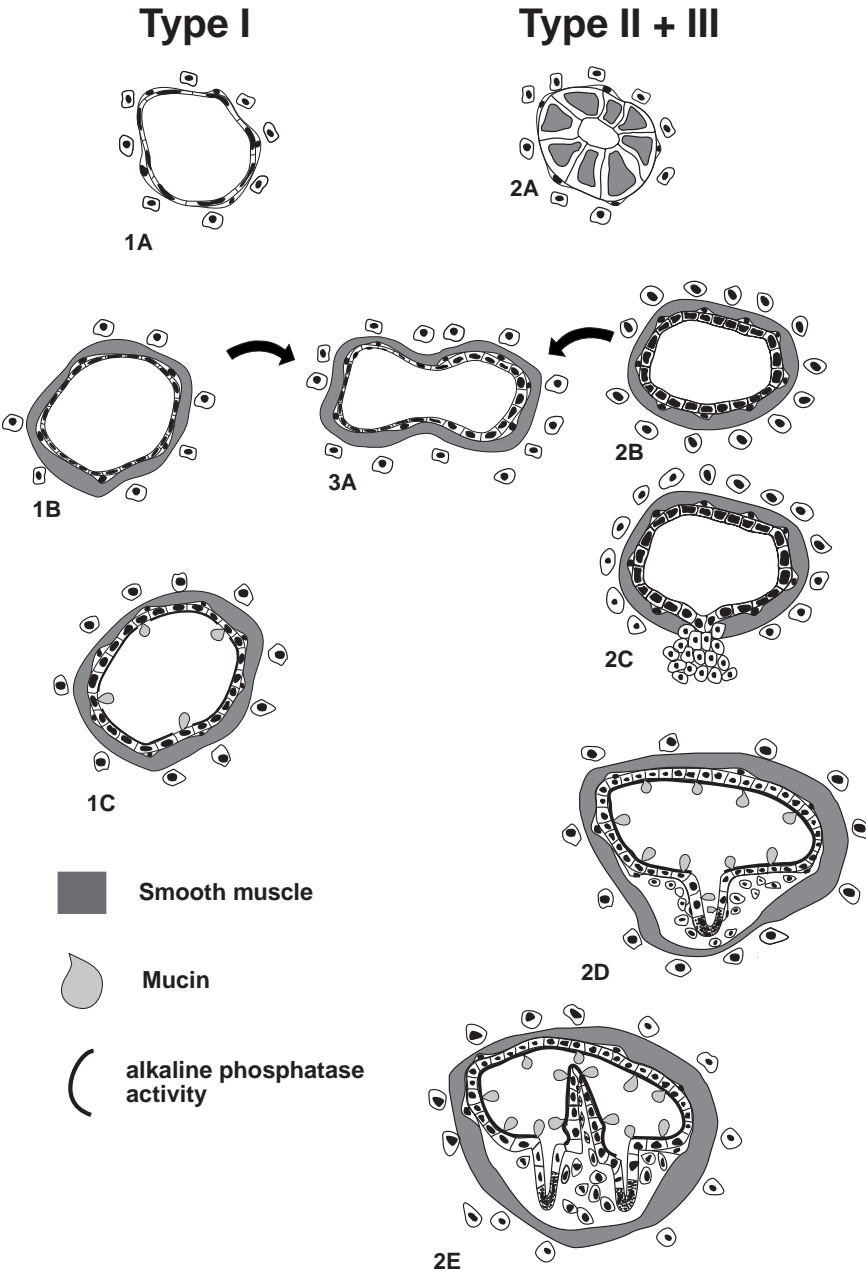


cell, the TA cell and the stem cell. Individual organoids contained a mixed population of both proliferative cell types, one cell type only, or only differentiated cells. The primary culture experiments support this idea as some organoids demonstrated a large proliferative capacity, as would be expected of stem cells, some a limited proliferative capacity as would be found in the TA cells, whilst others show no proliferation. A number of cultured organoids produce large sheets of undifferentiated cells on one side as well as smaller numbers of differentiated cells on the other, suggesting that cells of both proliferative capacities were present within the same organoid. Further support comes from the differentiated phenotype of cells from colonies with limited proliferation, whereas the colonies displaying a large proliferative capacity contain undifferentiated cells. To our knowledge, this is the first in vitro evidence that intestinal epithelial cells display

different proliferative and differentiating capacities which fit with current models of cell kinetics in the intestine (Potten and Loeffler, 1990).

We believe that the organoids maintain the same variations in proliferation and differentiation when grafted subcutaneously. Support for this comes from the early stages of cyst development. Type I cysts (Fig. 7) are lined with an undifferentiated flattened epithelium which proliferates, but only for a limited period. The cysts become filled with fluid before the epithelium converts to a columnar phenotype. This change in morphology is associated with differentiation of the epithelium as demonstrated by the presence of absorptive enterocytes, goblet cells and enteroendocrine cells. The conversion to a columnar differentiated epithelium is also associated with a decrease in cyst swelling. These properties are similar to those exhibited by TA cells in the small intestine.

**Fig. 7.** Model of neomucosal development from grafted organoids. All cysts are originally derived from single organoids. (1A) Type I cysts are derived from organoids containing only TA cells, which have a limited ability to proliferate and ultimately produce only terminally differentiated epithelial cells. (1B) These cysts increase in size as the cells proliferate to produce an undifferentiated flattened epithelial lining and are involved in vectorial fluid transport causing the cysts to swell. (1C) These cells then take on a columnar phenotype as they differentiate into absorptive enterocytes, goblet cells and enteroendocrine cells. This differentiation is associated with a decrease in cyst swelling caused by the inability of differentiated intestinal epithelium to transport fluid across their apical membrane. (2A) Type II cysts are derived from organoids containing stem cells only, while type III cysts are derived from organoids containing both stem cells and TA cells. Both types of cyst develop in a similar manner since it is the stem cell population which regulates cyst development. (2B) Undifferentiated epithelial cells proliferate to produce polarized columnar epithelial cells and cause the cyst to increase in size. (2C) At distinct sites along the epithelial monolayer (presumably the sites of intestinal stem cells), proliferating epithelium begins to migrate down towards the underlying mesenchyme. At the same time the mesenchyme migrates through layers of smooth muscle cells towards the epithelium. (2D) Cell migration continues leading to the generation of intestinal crypts, which contain Paneth cells at their base. Epithelial cell proliferation becomes restricted to these crypts. (2E) Regions of epithelium between the crypts protrude in towards the central lumen, leading to the generation of intestinal villi and full intestinal mucosal architecture. (3A) At any time after the formation of the original cysts, fusion may occur between neighbouring cysts. Fusion events lead to replacement of differentiated cells by the daughter cells of the stem cell population from type II and III cysts.



TA cells have a limited proliferative capacity and will terminally differentiate after 1-6 rounds of cell division (Potten and Loeffler, 1990). The undifferentiated TA cells of the crypt express the cystic fibrosis transmembrane conductance regulator gene (*Cfr*) (Cohn, 1994) and are involved in fluid secretion via cAMP-regulated chloride conductance, whereas the terminally differentiated cells of the villus do not (Welsh et al., 1982). This would explain the decrease in cyst swelling that accompanies the differentiation of the type I cyst epithelium. Experiments by Kirkland (1990) have shown that an undifferentiated intestinal cell line embedded in collagen can form cysts lined with columnar epithelium in vitro. Addition of various agents known to stimulate cAMP-mediated chloride conductance, and therefore fluid transport, cause the cysts to swell as they fill with fluid and the epithelium becomes flattened. Also, in vivo experiments by Del Buono et al. (1991) using the same cell line demonstrate that xenografts of these cells leads to the formation of cysts lined with flattened epithelium, which then converts into a columnar epithelium via signals derived from host mesenchymal cells. This conversion from a flattened to a columnar epithelium is also associated with differentiation of the cells.

Organoids containing a stem cell population should, if functional, be capable of reproducing an intestinal epithelial architecture. The development of type II and III cysts (Fig. 7) appears to confirm this. These cysts contain large undifferentiated epithelial-like cells, which develop into epithelium of a columnar phenotype. At distinct foci within the columnar epithelium, groups of cells appear to form small indentations in the epithelial lining. At the same time, mesenchymal cells from the distal side of the encircling smooth muscle appear to migrate towards this epithelium. The epithelial migration continues down into the mesenchyme to form an intestinal crypt surrounded by myofibroblasts. In the mature small intestine myofibroblasts also surround the lower portion of the crypt epithelium (Neal and Potten, 1981). This tight association with the proliferative compartment and in vitro data that demonstrates fibroblast-mediated regulation of intestinal epithelial cells (Haffen et al., 1983; Ratineau et al., 1997; Fritsch et al., 1997) suggests that these cells may be involved in regulating intestinal epithelial stem cell function. The process of crypt formation suggests that the regions of migrating epithelium contain at least one active stem cell. This conclusion is consistent with the identification of PCNA-positive cells within this population and from data showing that crypts form in the developing intestine from regions of proliferating epithelium in a similar manner (Alnafussi and Wright, 1982; Schmidt et al., 1988).

Once mature crypts have developed, new crypts are formed at the same region of the cyst. Crypt fission is the main mechanism used by the intestine to increase crypt number during the first few weeks of postnatal life and during intestinal epithelial regeneration (Li et al., 1994). During crypt fission, crypts enlarge and split in two to produce two daughter crypts; an example of this was found in one of the grafts (data not shown).

It is only after crypts have formed that alkaline phosphatase activity is detected in the cyst lumen. This activity is most likely derived from the apical brush border of absorptive enterocytes and suggests a degree of cell turnover with differentiated enterocytes being shed into the lumen, as occurs

in the small intestine (Creamer et al., 1961). After crypt formation, regions of epithelium between the crypts can be seen to protrude in towards the lumen. These protrusions develop into villi covered by differentiated epithelium, and a fully formed intestinal mucosa is generated. We believe this pattern of development supports our hypothesis that the grafted organoids maintain their proliferative and differentiation capacity.

Grafting experiments using marked cells revealed the hosts contribution to the neomucosal mesenchyme. Host stromal cells were recruited to the graft site and a proportion of these cells were induced to express  $\alpha$ -smooth muscle actin. This induction is most likely mediated by the intestinal epithelium since it has been shown that grafted intestinal endoderm is capable of inducing  $\alpha$ -smooth muscle actin not only in associated intestinal mesenchyme but also in skin fibroblasts (Kedinger et al., 1990).

The process of neomucosal generation from organoids is strikingly similar to intestinal regeneration (Inoue et al., 1988). During regeneration there is an initial response to seal the wound, termed epithelial restitution. This requires the flattening and spreading of the cells surrounding the wound to ensure the breach in the epithelial barrier is sealed quickly (Lacy, 1988). This is followed by the resumption of a columnar morphology by the cells. Proliferation takes place at distinct foci thought to be the location of surviving stem cells (Inoue et al., 1988) from which crypts are then formed and intestinal regeneration is completed with the formation of new villi.

We have shown here that an intestinal neomucosa can be generated efficiently from cell aggregates derived from neonatal mouse small intestine using a modified protocol. The neomucosa displays full crypt and villus architecture, containing all four differentiated epithelial cell types, as well as functional stem cells. The system described here provides a model for intestinal stem cell function and intestinal regeneration in the mouse. It is important that we have established this technique in the mouse as it has the big advantage that it can be manipulated to a much greater extent than in other mammals. In particular, the use of tissue derived from mice with specific gene mutations will aid our understanding of how specific genes allow stem cells to regulate proliferation, differentiation and crypt formation in the intestine.

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